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The role of interferon α/β in the induction of intestinal pathology in mice

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SUMMARY

We have investigated the role of interferon-alpha/beta (IFN-α/β) and IFN-dependent effector cells in causing enteropathy in mice. The IFN-inducer polyinosinic:polycytydylic acid (poly I:C) augmented the natural killer (NK) cell activation normally seen in neonatal (CBA × BALB/c)F_1 mice with graft-versus-host reaction (GVHR) and exacerbated the systemic and intestinal consequences of GVHR. Poly I:C itself produced a similar pattern of intestinal pathology when administered to normal mice. The effects of poly I:C on NK cell activity and intestinal architecture in normal mice could be reproduced by a single injection of purified IFN-α/β and the intestinal lesions caused by IFN-α/β were prevented by *in vivo* depletion of NK cells with anti-asialo GM1. These results indicate that IFN-α/β may play an important role in immunologically mediated enteropathies by virtue of its ability to activate NK cells.

INTRODUCTION

Graft-versus-host reactions (GVHR) in laboratory rodents have been used extensively as models of immunologically mediated tissue pathology[1,2] and a wide variety of effector mechanisms have been implicated.[2] However, there is now considerable evidence that inflammatory cytokines are responsible for the intestinal pathology which occurs in most forms of GVHR.[3,4] A more detailed understanding of the role of cytokines in intestinal GVHR might help the development of immunotherapeutic regimes for treatment of graft-versus-host disease (GVHD) after human bone marrow transplantation. In addition, it would clarify the pathogenesis of clinical enteropathies with mucosal pathology similar to that of GVHR.

That interferon-alpha/beta (IFN-α/β) may be one cytokine with a critical role in GVHR is suggested by the increased levels of IFN-α/β during systemic GVHR[5] and by the findings that the IFN-inducer, polyinosinic: polycytydylic acid (poly I:C), exacerbates systemic GVHR in mice.[6] As yet there is no direct evidence that IFN-α/β is involved in immune-mediated damage to specific target organs like the gut, but this possibility is supported by the fact that interferons are potent augmentors of natural killer (NK) activity (7) and that intestinal GVHR in mice can be prevented by *in vivo* depletion of the NK cells, which are recruited during this disease.[8,9]

In this paper we have directly tested the hypothesis that IFN-α/β is an enteropathic mediator by investigating the relationship between IFN-α/β, NK cell activation and intestinal damage, both in normal mice and in murine GVHR.

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MATERIALS AND METHODS

Mice

Adult CBA (H-2^k), BALB/c (H-2^d) and (CBA × BALB/c)F_1 mice were obtained from Harlan Olac (Bicester, Oxon, U.K.) and maintained under standard conditions until first used at age 6–8 weeks. Neonatal (CBA × BALB/c)F_1 mice were bred in the department and litters mixed at birth to obtain experimental groups of adequate size.

Induction and assessment of GVHR

A severe, acute GVHR was induced in 1–2-day-old neonatal mice by i.p. injection of 10^7 CBA spleen cells in 0·05 ml RPMI-1640 (Gibco BRL, Paisley, Renfrewshire, U.K.), as described previously.[10] Age-matched control animals received RPMI-1640 only. The intensity of systemic GVHR was assessed by measuring spleen weights, and was expressed as the spleen index.[12]

Treatment of mice with poly I:C

Normal adult mice were injected i.p. with 100 μg poly I:C (Sigma, Poole, Dorset, U.K.) diluted in 0·01 M NaOH, while neonatal mice received weight-related doses of 5 μg/g poly I:C 24 hr before induction of GVHR and on Day 18 of GVHR. Control mice received diluent alone.

Treatment of mice with IFN-α/β

Mice were injected i.p. with purified natural mouse IFN-α/β (Lee Biomolecular, San Diego, CA) in phosphate-buffered saline PBS containing 2% newborn calf serum (NCS; Gibco), while controls received mock IFN in diluent (Lee Biomolecular).

Treatment of mice with anti-asialo GM1 antiserum
Mice were injected intravenously with 0·3 ml rabbit anti-asialo GM1 antiserum (courtesy of Dr N. Hanna, S.K. & F. Research, Philadelphia, PA) diluted 1:50 in PBS, 24 hr before treatment with IFN-α/β. As a control, mice were injected with 0·3 ml normal rabbit serum (NRS).

Measurement of specific and non-specific cytotoxicity
Specific anti-host cytotoxic T-lymphocyte (CTL) activity was measured in the spleens of GVHR mice using P815 (H-2^d) mastocytoma cells, while NK cell activity was measured using YAC-1 target cells. As described in detail elsewhere,[9] target cells were labelled at 37° with 50 μCi $^{51}Cr/5 \times 10^6$ cells per ml and 100-μl aliquots containing 2×10^4 target cells then added to the wells of V-bottomed microtitre plates (Titertek, Flow Labs, Rickmansworth, U.K.). Spleen cells from two to three GVHR or control mice were pooled in RPMI-1640/5% NCS and 100-μl aliquots added to the microtitre plates to give effector:target (E:T) ratios of 50:1, 25:1 and 12·5:1. The plates were incubated for 4 hr at 37° and the percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100\%.$$

In all assays, 10% Triton X-100 (Sigma) was used to obtain maximum release. In NK assays, spontaneous release was measured using appropriate numbers of NK-inactive thymocytes from control F_1 mice, while control spleen cells were used to obtain spontaneous release in specific CTL assays.

Assessment of mucosal architecture
Villus and crypt lengths and crypt cell production rates (CCPR) were measured by microdissection of Feulgen-stained (Schiff Reagent; Difco) jejunum, as described previously.[11,12] Mice were killed at intervals of 20–100 min after injection of 7·5 mg/kg colchicine (Sigma) intraperitoneally to cause metaphase arrest, and pieces of jejunum were taken 10 cm from the pylorus. In each specimen 10 villi and crypts were measured using an eyepiece micrometer, and after counting the number of metaphases in 10 crypts the CCPR was calculated from the slope of the regression line of metaphase accumulation against time.

Statistical analysis
Groups of means and standard deviations were compared by Student's *t*-test, while crypt cell production rates were compared by covariance analysis.

RESULTS

Effects of poly I:C on acute GVHR in neonatal (CBA × BALB/c)F_1 mice

Poly I:C is a potent inducer of IFN-α/β production *in vivo*[13] and in our first experiments we examined the effects of poly I:C on the systemic and intestinal consequences of GVHR. To do this, we used a model of GVHR which we have studied extensively and in which a full range of intestinal damage occurs in neonatal hosts with a low basal NK cell activity.[10] The results described are representative of three replicate experiments in which poly I:C had essentially identical effects on GVHR.

As we have detailed previously,[10] 2-day-old (CBA × BALB/c)F_1 mice injected with 10^7 CBA spleen cells developed an acute GVHR, with clinical evidence of wasting, hunched posture and diarrhoea. In addition, all GVHR mice developed marked splenomegaly which was most intense on Day 12 and was significantly greater in GVHR mice treated with poly I:C (spleen index: $2{\cdot}4 \pm 0{\cdot}3$ versus $1{\cdot}6 \pm 0{\cdot}2$, $P < 0{\cdot}02$, four mice/group).

Effects of poly I:C on cell-mediated cytotoxicity during GVHR in neonatal mice

As we have shown previously,[10] control mice of this age had virtually no NK activity when examined on Day 9 of the experiment, and there was little evidence of NK cell activity in GVHR mice or in control mice given poly I:C (Fig. 1a). However, GVHR mice treated with poly I:C on Day 8 had readily detectable NK cell activity, which was very marked in comparison to the other groups.

Thus, poly I:C and acute GVHR had a synergistic ability to activate NK cells in the early stages of GVHR. In contrast, poly I:C had quite opposite effects on specific CTL activity in GVHR. Although both groups of GVHR mice had detectable CTL activity by Day 12, this was greater in control GVHR mice than in the mice which had received poly I:C (Fig. 1b, 8·5% versus 2·7% lysis at 50:1 E:T ratio). Previous work has shown these CTL to be $CD8^+$, host-specific T cells.[10] Thus the effects of poly I:C on GVHR were not due to increased CTL activity.

Effects of poly I:C on intestinal GVHR in neonatal mice

By Day 9 of the experiment, untreated mice with GVHR had developed significant villus atrophy and crypt hypertrophy compared with controls (Fig. 2), confirming previous work on this model (10). Similar, but more severe, intestinal pathology was found in GVHR mice given poly I:C, with significantly shorter villi and longer crypts than in untreated mice with GVHR (Fig. 2). Furthermore, whereas untreated GVHR mice at this time had a CCPR which was not significantly different from that of controls, poly I:C-treated mice with GVHR had a

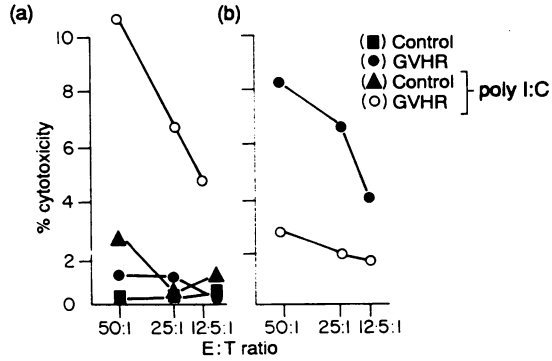

Figure 1. Effects of poly I:C on the development of specific and non-specific cell-mediated cytotoxicity in neonatal (CBA × BALB/c)F_1 mice with GVHR. (a) NK cell activity in the spleens of poly I:C-treated or control mice with GVHR and in appropriate controls. (b) Anti-host-specific CTL activity measured against P815 targets in spleens of poly I:C-treated and control mice with GVHR. Results shown are percentage at different E:T ratios, using spleen cells pooled from three mice/group on Day 9 (NK) or 12 (CTL) of GVHR.

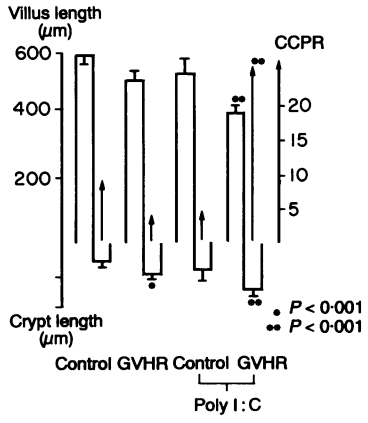

Figure 2. Effects of poly I:C on intestinal GVHR in neonatal (CBA × BALB/c)F_1 mice. Mucosal architecture in the jejunum of poly I:C-treated or control mice with GVHR on Day 9 of GVHR, and in appropriate controls. Bars represent mean villus and crypt lengths ± 1 SD, while arrows show the CCPR. Results are from four mice/group.

Table 2. Effects of IFN-α/β on NK activity in normal, adult (CBA × BALB/c)F_1 mice

Time post-treatment (hr)	Dose of IFN-α/β (U)	% cytotoxicity at 50:1 E:T ratio	
		IFN-α/β	Control
24	0	—	35·4
	10^3	28·7	—
	10^4	52·6	—
	10^5	67·8	—
24	10^5	36·1	10·9
48	10^5	21·8	6·6
72	10^5	15·1	11·5

Time-course and dose-dependency of NK cell activation in the spleens of IFN-α/β-treated and control mice. Results shown are percentage cytotoxicity at 50:1 E:T ratio, using spleen cells pooled from three mice/group.

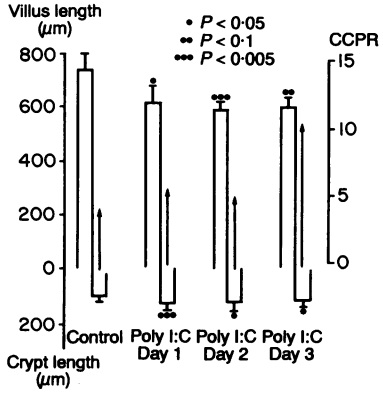

Figure 3. Induction of intestinal pathology by administering poly I:C to normal (CBA × BALB/c)F_1 mice. Mucosal architecture in the jejunum of poly I:C-treated or control mice, 1, 2 and 3 days after a single injection of 100 μg poly I:C. Bars represent mean villus and crypt lengths ± 1 SD, while arrows shown the CCPR for five mice/group.

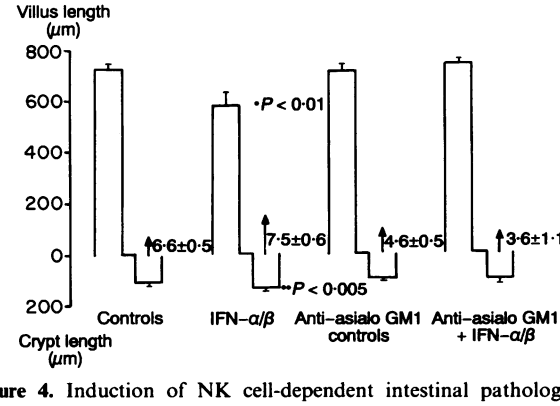

Figure 4. Induction of NK cell-dependent intestinal pathology by administration of IFN-α/β to normal adult mice. Mucosal architecture in the jejunum of mice given 10^5 U IFN-α/β ip 24 hr before, with or without pretreatment with anti-asialo GM1 antiserum and in appropriate controls. Bars represent mean villus and crypt lengths ± 1 SD for five animals/group.

Table 1. Effects of poly I:C on NK activity in normal, adult (CBA × BALB/c)F_1 mice

Time post-treatment (hr)	% cytotoxicity					
	Control			Poly I:C		
	50:1	25:1	12·5:1	50:1	25:1	12·5:1
24	16·2	13·8	9·1	59·1	48·2	42·6
48	19·1	16·5	10·9	64·4	55·3	47·7
72	20·2	17·1	13·4	46·4	37·8	29·6

NK activity in the spleens of mice given 100 μg poly I:C at intervals and in controls. Results shown are percentage cytotoxicity at three E:T ratios, using spleen cells pooled from three mice/group.

very marked increase in CCPR (26·4 ± 3·0 versus 4·8 ± 1·7 for untreated GVHR, $P < 0·001$).

Effects of poly I:C on mucosal architecture in normal adult mice

The findings described above indicate that enhancing the production of IFN-α/β is associated with the development of more severe enteropathy in GVHR. In view of the non-specific nature of these mediators, we reasoned that it might be possible to induce enteropathy in normal animals by augmenting IFN-α/β activity to sufficiently high levels. In addition, as poly I:C-treated mice with GVHR had the unusual feature of significant NK cell activation, and as depletion of NK cells *in vivo* ameliorates intestinal GVHR,[9] we wished to test the hypothesis that the enteropathic effects of IFN-α/β were mediated through its well-known ability to activate NK cells. There was no

significant evidence of intestinal pathology in normal neonatal mice given poly I:C (Fig. 2). However, these animals have very low basal NK cell activity and have a limited capacity to produce IFN-α/β.[14] Therefore, we thought it more appropriate to continue these studies in mature mice with intact NK cell function and first examined the effects of poly I:C on the intestine of adult (CBA × BALB/c)F_1 mice.

Normal adult mice given 100 μg poly I:C developed significant villus atrophy, which was present by Day 1 and remained until at least 3 days post-injection (Fig. 3). Poly I:C-treated mice also had significant crypt hypertrophy at all times, although this appeared most marked on Day 1. There were also significant increases in CCPR after treatment with poly I:C (Fig. 3). In parallel, the mice given 100 μg of poly I:C had elevated levels of NK activity within 24 hr, which continued to increase at 48 hr and were still well above control levels at 72 hr (Table 1).

Thus, poly I:C produces a significant intestinal lesion in normal adult mice and this parallels the enhancement of NK cell activity in the same animals. Identical findings have been made in three subsequent experiments.

Effects of IFN-α/β on mucosal architecture of adult mice

The next experiments were designed to establish that the effects of poly I:C on mucosal architecture reflected its ability to induce production of IFN-α/β and to determine the role of NK cells in this phenomenon.

Appropriate antibodies were not available to deplete poly I:C-treated mice of IFN-α/β and therefore we examined the effects of administering purified cytokine to normal, adult mice. Once again, several studies were performed to examine these effects and the results shown are representative of three experiments which gave similar results. Initial experiments were performed to confirm that IFN-α/β augmented NK cell activity *in vivo* and to determine the optimal times and doses to use in studies of intestinal pathology. Intraperitoneal administration of IFN-α/β markedly enhanced splenic NK cell activity *in vivo* and these effects were maximal 24 hr after injection of 10^5 U cytokine (Table 2). In parallel, this dose of IFN-α/β induced significant villus atrophy and crypt hypertrophy 24 hr after i.p. injection into normal mice (Fig. 4). These effects were abolished by treating mice with anti-asialo GM1 antibody, a regime known to deplete NK cells.[9] These results support the view that stimulation of IFN-α/β production underlies the pathological effects of poly I:C on the intestine. In addition, our data indicate that the enteropathic action of IFN-α/β is a NK cell-dependent phenomenon.

DISCUSSION

The results presented here support the view that IFN-α/β may play an important role in immunologically mediated enteropathy and indicate that this depends on the ability of IFN to activate NK cells *in vivo*. The first piece of evidence which suggests an enteropathic effect for IFN-α/β was that administration of poly I:C exacerbated the intestinal consequences of GVHR in neonatal mice. Our finding that poly I:C-treated mice had more splenomegaly in GVHR confirms previous reports on systemic GVHR in mice[13] and we have now extended these studies by demonstrating that poly I:C also markedly exacerbated the intestinal pathology which occurs during acute GVHR, with more intense villus atrophy and crypt hyperplasia. Interestingly, poly I:C itself caused a similar pattern of intestinal pathology within 24 hr of administration to normal mice, an effect which was only seen in mature rather than neonatal animals. As others have shown,[13] poly I:C had a marked ability to enhance NK cell activity *in vivo* and the pathological effects of poly I:C correlated with the degree of NK cell activation in the two models. Thus, intestinal damage was associated with enhanced NK cell activity in both normal and GVHR mice given poly I:C, while the differential effects of poly I:C on intestinal pathology in normal adult or neonatal mice correlated well with their basal NK cell levels. In contrast, enhanced CTL activity is unlikely to account for the enteropathic effects of poly I:C in GVHR, as poly I:C-treated mice had lower than normal specific CTL activity. These results are further evidence against a pathogenic role for CTL in intestinal GVHR and are more consistent with a critical role for non-specific mechanisms.

As the best characterized property of poly I:C is to enhance the production of interferons, and particularly that of α and β IFN,[5,15] we investigated directly whether IFN-α/β could account for the enteropathic effects of poly I:C. As anticipated, mice given purified IFN-α/β displayed markedly enhanced splenic NK cell activity, which had appeared by 24 hr and persisted until at least 48 hr. In parallel, IFN-treated mice developed significant villus atrophy and crypt hypertrophy in their small intestine. These features were identical to those found in poly I:C-treated animals and are consistent with the hypothesis that the pathological effects of this agent are caused by IFN α/β. This requires direct proof by depleting poly I:C-treated mice with appropriate IFN-specific antibodies.

A consistent feature of our studies was the relative speed at which a full spectrum of intestinal pathology developed after one injection of either poly I:C or IFN-α/β. Previous studies using intestinal GVHR as a model of experimental enteropathy have demonstrated that the intestinal damage develops progressively, with crypt hyperplasia preceding villus atrophy.[10,16] These findings have led to the concept that dividing crypt cells provide the principal target for the immune effector mechanisms. However, in the current study, fully developed villus atrophy and crypt hyperplasia were usually observed together, within 24 hr after injection of poly I:C or IFN-α/β. We believe this difference probably reflects the fact that a developing immune response such as that found in GVHR will take some time to produce sufficient mediators to cause a complete enteropathy, whereas high doses of purified cytokine may damage the intestine very rapidly. We are currently performing more detailed dose–response and time–course studies to establish the evolution of IFN-α/β-induced enteropathy and hence identify the primary target of its actions in the gut.

Our evidence that IFN-α/β plays a critical role in immunopathology is supported not only by our findings that poly I:C exacerbates intestinal GVHR but also by the fact that there are increased levels of IFN-α/β in the serum and lymphoid tissues of mice with GVHR.[5,13,16] Clearly, IFN-α/β is not the only mediator of intestinal GVHR, as neonatal mice develop marked enteropathy despite having virtually no NK cell activation. However, our results show that IFN-α/β contributes to the severity of this lesion. It could produce its effects on the gut either by a direct action on epithelial cells or via activation of

non-specific effector mechanisms. The enteropathic effects of both poly I:C and IFN-α/β were accompanied by enhanced NK cell activity *in vivo*. Furthermore, the intestinal lesions induced by IFN-α/β were prevented by treating mice with anti-asialo GM1 antibody, a procedure which produces selective and profound depletion of NK cells, both systemically and in the gut itself.[9,17,18] Together, these findings demonstrate that activation of NK cells is required for IFN-α/β to cause intestinal damage and are consistent with the view that IFN-α/β may not have direct effects on epithelial cells themselves. Depletion of NK cells by anti-asialo GM1 also prevents the enteropathy and other features of GVHR in mice with GVHR,[9,18,19] indicating that IFN-dependent activation of NK cells may also be important in this form of immunopathology. The mechanisms by which activated NK cells could produces intestinal damage remain to be determined, but could reflect a direct cell-mediated cytotoxic activity. Alternatively, NK cells might produce further soluble mediators which induce epithelial cell injury such as TNF-α, which is produced by NK cells[20] and is known to be critical for the enteropathy of murine GVHR.[4]

The stimulus for, and source of, IFN-α/β production in GVHR is also unknown. Nevertheless, we would propose that this mediator is produced by macrophages which have been stimulated by activated T lymphocytes. We have shown previously that IFN-γ is essential for enteropathy in GVHR,[3] and others have shown that increased production of IFN-β by macrophages in GVHR is dependent on IFN-γ.[6] Together, these findings indicate that a complex cascade of immunological mediators is involved in immunologically mediated enteropathy and we are currently investigating directly the interactions between different cytokines and effector cells in different experimental models. In addition, it will be important to assess the clinical relevance of our work by determining whether IFN-α/β production is increased in the mucosa of patients with intestinal disease, as has been shown recently for TNF-α.[21] Identification of enteropathic mediators under clinical and experimental conditions may ultimately lead to cytokine-specific immunotherapy for a number of clinical disorders.

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